UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/714,310	11/14/2003	Zhidong Chen	12475/50102	5753

26646 7590 06/27/2006

KENYON & KENYON LLP
ONE BROADWAY
NEW YORK, NY 10004

EXAMINER	
GIBBS, TERRA C	

ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 06/27/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/714,310	CHEN ET AL.	
	Examiner	Art Unit	
	Terra C. Gibbs	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *02 May 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-42* is/are pending in the application.

4a) Of the above claim(s) *1-24 and 33-42* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *25-32* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *14 November 2003* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *February 9, 2006*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☒ Other: *sequence search alighments*.

DETAILED ACTION

This Office Action is a response to Applicant's Election filed May 2, 2006.

Claims 1-42 are pending in the instant application.

Claims 1-24 and 33-42 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. It is further noted that SEQ ID NOs: 1-16, 19, 20, 22-31, 33, 34, and 36 are also withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on May 2, 2006.

Claims 25-32 have been examined on the merits.

Election/Restrictions

Applicant's election without traverse of Group XIII, claims 25-32 in the reply filed May 2, 2006 is acknowledged. Applicant's further election of SEQ ID NO:32 is also acknowledged.

The requirement is still deemed proper and is therefore made FINAL.

Information Disclosure Statement

Applicant's information disclosure statement filed February 9, 2006 is acknowledged. The submission is in compliance with the provisions of 37 CFR §1.97.

Accordingly, the Examiner has considered the information disclosure statement, and a signed copy is enclosed herewith.

Specification

The specification is objected to because the specification at page 14 contains embedded hyperlinks and/or other forms of browser-executable code that are impermissible and must be deleted. The attempt to incorporate subject matter into the patent application by reference to a hyperlink and/or other forms of browser-executable code is considered to be an improper incorporation by reference. See MPEP 608.01(p), paragraph I regarding incorporation by reference. Furthermore, if the application should issue and be placed on the Office web page, the URL may be interpreted as a valid HTML code and become a live web link, transferring a user to a commercial web site. Office policy does not permit the Office to link to any commercial site because the Office exercises no control over the organization, views or accuracy of the information contained on these outside sites.

The above is an example and is not intended to indicate that the Examiner has made an exhaustive review of the application. Applicants are urged to review the disclosure and remove embedded hyperlinks and/or other forms of browser-executable codes that are impermissible.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 25-32 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 25 recites, "[A]n isolated oligonucleotide comprising a sequence of at least 8 contiguous nucleobases which is substantially identical or complementary to at least a portion of SEQ ID NO:21 or an RNA sequence corresponding thereto". It is unclear whether the *isolated oligonucleotide* or the *at least 8 contiguous nucleobases* is/are substantially identical or complementary to at least a portion of SEQ ID NO:21 or an RNA sequence corresponding thereto. Clarification is required. It is noted that claims 26-32 are rejected for their dependency therein.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 25-28 and 32 are rejected under 35 U.S.C. 102(b) as being anticipated by Koster et al. [U.S. Patent No. 6,043,031] ('031).

Claim 25 is drawn to an isolated oligonucleotide comprising a sequence of at least 8 contiguous nucleobases which is substantially identical or complementary to at least a portion of SEQ ID NO:21 or an RNA sequence corresponding thereto. Claims 26-28 and 32 are dependent on claim 25 and include all the limitations of claim 25 with the further limitations wherein the sequence is substantially identical or complementary to SEQ ID NO:17 or an RNA sequence corresponding thereto; wherein the sequence is substantially identical or complementary to SEQ ID NO:32, or an RNA sequence corresponding thereto; wherein the substantially identical or complementary sequence is about 10-23 nucleobases in length; and a composition comprising the oligonucleotide of claim 25 and a pharmaceutically acceptable carrier, diluent or adjuvant. It is noted that the instant specification does not explicitly define the term, "substantially identical". However, the instant specification at page 14, lines 16-20 defines, "[T]wo DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules". The instant specification explicitly defines, "[H]omology" refers to the percent of identity between at least two oligonucleotides or polypeptides". Given the fact that the instant specification has defined the term, "substantially homologous" and has defined the term "homology" to infer identity, the Examiner is interpreting the term, "substantially identical" to be synonymous with "substantially homologous", as defined in the instant specification.

'031 discloses a CFTR gene exon 10 PCR primer with the following sequence: 5'-GTGTGAAGGGTTCATATG-3' (see EX10-R or SEQ ID NO:2). It is noted that SEQ ID NO:2 disclosed by '031 is complementary to an 8-nucleobase portion of SEQ ID NO:17. For example, SEQ ID NO:2 is complementary to nucleobases 1-8 of SEQ ID NO:17 of the instant invention. It is further noted that SEQ ID NO:2 disclosed by '031 is complementary to an 8-nucleobase portion of SEQ ID NO:21. For example, SEQ ID NO:2 is complementary to nucleobases 5-12 of SEQ ID NO:21 of the instant invention (see attached sequence alignment). It is also noted that SEQ ID NO:2 disclosed by '031 is identical to an 8-nucleobase portion of SEQ ID NO:32. For example, SEQ ID NO:2 is 100% identical to nucleobases 10-17 of SEQ ID NO:32 of the instant invention. It is noted that SEQ ID NO:2 disclosed by '031 was used in a PCR Amplification reaction using standard PCR conditions and thus the buffers included in the reaction mixture constitute pharmaceutically acceptable carriers as recited in claim 32.

Therefore, Koster et al. [U.S. Patent No. 6,043,031] ('031) anticipates claims 25-28 and 32.

Claims 25-28 and 32 are rejected under 35 U.S.C. 102(b) as being anticipated by Stoler et al. [U.S. Patent No. 5,912,147] ('147).

The claims are as described above in the 35 U.S.C. 102(b) as being anticipated by Koester et al. Further, the Examiner's interpretation of the term, "substantially identical" is as discussed and detailed above.

'147 discloses the use of a 3'-anchored primer $(CA)_x$ primer in amplification of genomic DNA from tumors with the following sequence:
5'-CGCGCGCGGT-3' (see primer 15 or SEQ ID NO:15). It is noted that SEQ ID NO:15 disclosed by '147 is complementary to an 8-nucleobase portion of SEQ ID NO:17. For example, SEQ ID NO:15 is complementary to nucleobases 7-14 of SEQ ID NO:17 of the instant invention. It is further noted that SEQ ID NO:15 disclosed by '147 is complementary to an 8-nucleobase portion of SEQ ID NO:21. For example, SEQ ID NO:15 is complementary to nucleobases 11-18 of SEQ ID NO:21 of the instant invention (see attached sequence alignment). It is also noted that SEQ ID NO:15 disclosed by '147 is identical to an 8-nucleobase portion of SEQ ID NO:32. For example, SEQ ID NO:15 is 100% identical to nucleobases 4-11 of SEQ ID NO:32 of the instant invention. It is noted that SEQ ID NO:15 disclosed by '147 was used in an inter-SSR PCR amplification reaction using standard amplification conditions and thus the buffers included in the reaction mixture constitute pharmaceutically acceptable carriers as recited in claim 32.

Therefore, Stoler et al. [U.S. Patent No. 5,912,147] ('147) anticipates claims 25-28 and 32.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 25-28 and 30-32 are rejected under 35 U.S.C. 103(a) as being unpatentable over either Koester et al. [U.S. Patent No. 6,043,031] ('031) or Anderson et al. [U.S. Patent No. 5,912,147] ('147), in further view of Skerra, A. (Nucleic Acids Research, 1992 Vol. 20:3551-3554).

Claim 25 is drawn to an isolated oligonucleotide comprising a sequence of at least 8 contiguous nucleobases which is substantially identical or complementary to at least a portion of SEQ ID NO:21 or an RNA sequence corresponding thereto. Claims 26-28 and 30-32 are dependent on claim 25 and include all the limitations of claim 25 with the further limitations wherein the sequence is substantially identical or complementary to SEQ ID NO:17 or an RNA sequence corresponding thereto; wherein the sequence is substantially identical or complementary to SEQ ID NO:32, or an RNA sequence corresponding thereto; wherein the substantially identical or complementary sequence is about 10-23 nucleobases in length; wherein the oligonucleotide comprises an internucleotide linkage, wherein the internucleotide linkage is a phosphorothioate linkage; and a composition comprising the oligonucleotide of claim 25 and a

pharmaceutically acceptable carrier, diluent or adjuvant. It is noted that the instant specification does not explicitly define the term, "substantially identical". However, the instant specification at page 14, lines 16-20 defines, "[T]wo DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules". The instant specification explicitly defines, "[H]omology" refers to the percent of identity between at least two oligonucleotides or polypeptides". Given the fact that the instant specification has defined the term, "substantially homologous" and has defined the term "homology" to infer identity, the Examiner is interpreting the term, "substantially identical" to be synonymous with "substantially homologous", as defined in the instant specification.

'031 teaches a CFTR gene exon 10 PCR primer with the following sequence:

5'-GTGTGAAGGGTTCATATG-3' (see EX10-R or SEQ ID NO:2). It is noted that SEQ ID NO:2 taught by '031 is complementary to an 8-nucleobase portion of SEQ ID NO:17. For example, SEQ ID NO:2 is complementary to nucleobases 1-8 of SEQ ID NO:17 of the instant invention. It is further noted that SEQ ID NO:2 taught by '031 is complementary to an 8-nucleobase portion of SEQ ID NO:21. For example, SEQ ID NO:2 is complementary to nucleobases 5-12 of SEQ ID NO:21 of the instant invention. It is also noted that SEQ ID NO:2 taught by '031 is identical to an 8-nucleobase portion of SEQ ID NO:32. For example, SEQ ID NO:2 is 100% identical to nucleobases 10-17 of SEQ ID NO:32 of the instant invention. It is noted that SEQ ID NO:2 taught by '031 was used in a PCR Amplification reaction using standard PCR conditions and thus the

buffers included in the reaction mixture constitute pharmaceutically acceptable carriers as recited in claim 32.

'147 teaches the use of a 3'-anchored primer $(CA)_x$ primer in amplification of genomic DNA from tumors with the following sequence:
5'-CGCGCGCGGT-3' (see primer 15 or SEQ ID NO:15). It is noted that SEQ ID NO:15 taught by '147 is complementary to an 8-nucleobase portion of SEQ ID NO:17. For example, SEQ ID NO:15 is complementary to nucleobases 7-14 of SEQ ID NO:17 of the instant invention. It is further noted that SEQ ID NO:15 taught by '147 is complementary to an 8-nucleobase portion of SEQ ID NO:21. For example, SEQ ID NO:15 is complementary to nucleobases 11-18 of SEQ ID NO:21 of the instant invention. It is also noted that SEQ ID NO:15 taught by '147 is identical to an 8-nucleobase portion of SEQ ID NO:32. For example, SEQ ID NO:15 is 100% identical to nucleobases 4-11 of SEQ ID NO:32 of the instant invention. It is noted that SEQ ID NO:15 taught by '147 was used in an inter-SSR PCR amplification reaction using standard amplification conditions and thus the buffers included in the reaction mixture constitute pharmaceutically acceptable carriers as recited in claim 32.

Neither '031 nor '147 teach wherein the oligonucleotide comprises a phosphorothioate internucleotide linkage.

Skerra, A. teaches phosphorothioate-modified primers improve the amplification of DNA sequences by DNA polymerase with proofreading activity (see Abstract). Skerra, A. teaches the introduction of single phosphorothioate bond at the 3' termini of the PCR primer protects the oligodeoxynucleotide from exonucleolytic attack leading to

specific and efficient amplification of DNA.

It would have been *prima facie* obvious to one of ordinary skill in the art to make an isolated oligonucleotide comprising a sequence of at least 8 contiguous nucleobases which is substantially identical or complementary to at least a portion of SEQ ID NO:21, and have the oligonucleotide further comprise a phosphorothioate internucleotide linkage using the teachings of '031 or '147 and following the method and motivation of Skerra, A.

One of ordinary skill in the art would have been motivated to make an isolated oligonucleotide comprising a sequence of at least 8 contiguous nucleobases which is substantially identical or complementary to at least a portion of SEQ ID NO:21, and have the oligonucleotide further comprise a phosphorothioate internucleotide linkage since Skerra taught the introduction of single phosphorothioate bond on the PCR primer protects the oligodeoxynucleotide from exonucleolytic attack leading to specific and efficient amplification of DNA.

One of ordinary skill in the art would have expected success at making an isolated oligonucleotide comprising a sequence of at least 8 contiguous nucleobases which is substantially identical or complementary to at least a portion of SEQ ID NO:21, and have the oligonucleotide further comprise a phosphorothioate internucleotide linkage because the oligonucleotides of '031 and '147 are primers used for PCR and Skerra taught the successful design and use of phosphorothioate-modified primers in the amplification of DNA sequences during PCR.

Therefore the invention would have been *prima facie* obvious to one of ordinary

skill in the art at the time the invention was filed.

Conclusion

No claims are allowable.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Terra C. Gibbs whose telephone number is 571-272-0758. The examiner can normally be reached on 9 am - 5 pm M-F.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Peter Paras can be reached on 571-272-4517. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

tcg
June 24, 2006

Terra C. Gibbs

Nucleic Acids Research, Vol. 20, No. 14 3551–3554

Phosphorothioate primers improve the amplification of DNA sequences by DNA polymerases with proofreading activity

Arne Skerra
Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Straße 7, W-6000 Frankfurt/Main 71, Germany

Received June 15, 1992; Accepted June 25, 1992

ABSTRACT

Two thermostable DNA polymerases with proofreading activity—*Vent* DNA polymerase and *Pfu* DNA polymerase—have attracted recent attention, mainly because of their enhanced fidelities during amplification of DNA sequences by the polymerase chain reaction. A severe disadvantage for their practical application, however, results from the observation that due to their 3′ to 5′ exonuclease activities these enzymes degrade the oligodeoxynucleotides serving as primers for the DNA synthesis. It is demonstrated that this exonucleolytic attack on the primer molecules can be efficiently prevented by the introduction of single phosphorothioate bonds at their 3′ termini. This strategy, which can be easily accomplished using routine DNA synthesis methodology, may open the way to a widespread use of these novel enzymes in the polymerase chain reaction.

INTRODUCTION

Apart from its application in the detection and analysis of novel nucleic acid sequences the polymerase chain reaction (PCR) is gaining extended use during the routine manipulation of already well characterized DNA sequences (for a recent review see ref. 1). In particular in the field of protein engineering PCR frequently replaces previously established techniques for the cloning and subcloning of structural genes, the construction of expression plasmids, and the introduction of site-specific mutations. However, it has been recognized that the occurence of random mutations during DNA amplification caused by the limited fidelity of *Taq* DNA polymerase—up to now the enzyme almost exclusively used for PCR—constitutes a general problem.

Recently, two novel thermostable DNA polymerases were introduced apart from *Thermus aquaticus* DNA polymerase: *Vent* DNA polymerase from *Thermococcus litoralis* manufactured by New England Biolabs (2) and *Pfu* DNA Polymerase from *Pyrococcus furiosus* manufactured by Stratagene (3). Both enzymes have been reported to exhibit 3′ to 5′ exonuclease or proofreading activity resulting in a significantly increased base substitution fidelity compared to *Taq* polymerase, an enzyme not displaying this property. In addition, it was shown that—in contrast to *Taq* DNA polymerase—*Vent* DNA polymerase generates PCR products with blunt ends, thus permitting their direct cloning into a blunt-cut vector without the need for further modification steps (4).

Although these properties make the practical application of both thermostable proofreading DNA polymerases highly desirable, published investigations which make use of these novel molecular biology reagents have been rare so far. This is probably due to the observation that the two enzymes show a marked dependence on the design of the primer oligodeoxynucleotides used for the PCR reaction, both in terms of yield and specificity of the product. This behaviour of *Pfu* DNA polymerase and *Vent* DNA polymerase represents a strong contrast to *Taq* DNA polymerase, an enzyme which leads to consistent amplification results over a wide range of experimental setups, even under non-optimized standard reaction conditions.

Observations made in the author's laboratory during the use of *Pfu* DNA polymerase and *Vent* DNA polymerase with different combinations of template nucleic acids and primer oligodeoxynucleotides lead, together with a technical note on *Vent* DNA polymerase released by the manufacturer (2), to the following hypothesis for the explanation of the experimental observations.

The thermostable proofreading DNA polymerases act on single-stranded primer molecules present in the reaction solution, degrading them from the 3′ terminus and leaving a limit 5′ residual product of approximately 15 nucleotides in length (2). If still complementary to the template, these shortened primer molecules in principle are able to anneal to the template, at least at lower temperatures, though with reduced specificity. If, however, one of the primer oligodeoxynucleotides was designed such that only its 3′ terminus matches to the template but its 5′ terminal sequence does not—which is frequently the case when introducing novel restriction sites for cloning purposes or when even changing the nucleic acid sequences flanking, e. g., the coding region of a gene—the degraded primer does not give rise to a PCR product at all.

Therefore, a much better performance of the thermostable proofreading DNA polymerases was to be expected if a way was found to prevent the oligodeoxynucleotide primers from this exonucleolytic attack. As will be demonstrated here, this protection can be achieved simply by the introduction of a single phosphorothioate bond during synthesis of the oligodeoxynucleotide, leading indeed to the anticipated positive

effect on the specific and efficient amplification of DNA sequences with *Pfu* DNA polymerase and *Vent* DNA polymerase.

MATERIALS AND METHODS

The primer oligodeoxynucleotides were synthesized on a model 392A-05 Applied Biosystems automated DNA synthesizer using standard phosphoamidite solid phase chemistry (5). Phosphorothioate bonds were selectively introduced by oxidizing the intermediary phosphite triester with either tetraethylthiuram disulfide (6) supplied by Applied Biosystems or the Beaucage thiolating reagent (7) purchased from Pharmacia. Both reagents were found to perform similarly well. However, care had to be taken that the thio-oxidation was complete because otherwise a mixture of two reaction products, one with the phosphorothioate bond and one with the usual phosphodiester bond, was obtained. Suitable synthesis cycle programs will be made available from the author upon request. The oligodeoxynucleotides were purified by denaturing polyacrylamide gel electrophoresis and finally quantified by UV absorption at 260 nm (cf. ref. 8).

The polymerase chain reactions were carried out in a total volume of 50 µl with 5 µl 10× reaction buffer, 4 µl dNTP solution containing 2.5 mM of each dATP, dCTP, dGTP, dTTP (Pharmacia), 2.5 µl of each primer at a concentration of 10 µM, and approximately 100 pg pASK30 supercoiled plasmid DNA (9) as the template. The reagents were mixed, overlaid with paraffin oil, and heated for approximately 2 min at 94°C. Thermocycling was started after addition of the DNA polymerase. The following reaction buffers and amounts of the different enzymes were used:

i) 0.5 µl *Taq* DNA polymerase (5 u/µl; Perkin Elmer Cetus) with a standard 10× reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0, 15 mM $MgCl_2$, 0.1% gelatin, and 1% Triton X-100);

ii) 1 µl *Pfu* DNA Polymerase (2.5 u/µl; Stratagene) with the 10× reaction buffer #1 (200 mM Tris-HCl pH 8.2, 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1% Triton X-100, 100 ng/µl BSA) supplied by the manufacturer of the DNA polymerase;

iii) 1 µl *Vent* DNA Polymerase (1 u/µl; New England Biolabs) with the 10× standard reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8.8, 20 mM $MgSO_4$, 1% Triton X-100) supplied by the manufacturer of the DNA polymerase.

Irrespective of the DNA polymerase, the PCR amplification was carried out using 25 thermocycles with 60 s at 94°C (strand separation), 60 s at 55°C (annealing), and 90 s at 72°C (second strand synthesis), followed by a final incubation at 60°C for 5 min. In the case of *Vent* DNA polymerase, which was used at lower activity, 1 ng template DNA was amplified with 20 thermocycles and an annealing temperature of 65°C. After addition of loading buffer, 10 µl of each of the resulting solutions was directly applied to a 1% agarose gel containing 1µg/ml ethidium bromide in TBE buffer (8).

RESULTS AND DISCUSSION

Starting from the assumptions outlined above it was sought to prevent the 3′ terminal hydrolysis of the PCR primers by introducing a single phosphorothioate bond at the first 3′ terminal internucleotide linkage during synthesis of the oligodeoxynucleotide. The phosphorothioate bond was described to be a much less favoured substrate to nuclease activity than the naturally occuring phosphodiester bond (10). In order to test whether this chemical modification can improve the PCR reaction with thermostable proofreading DNA polymerases the following experimental setup was used.

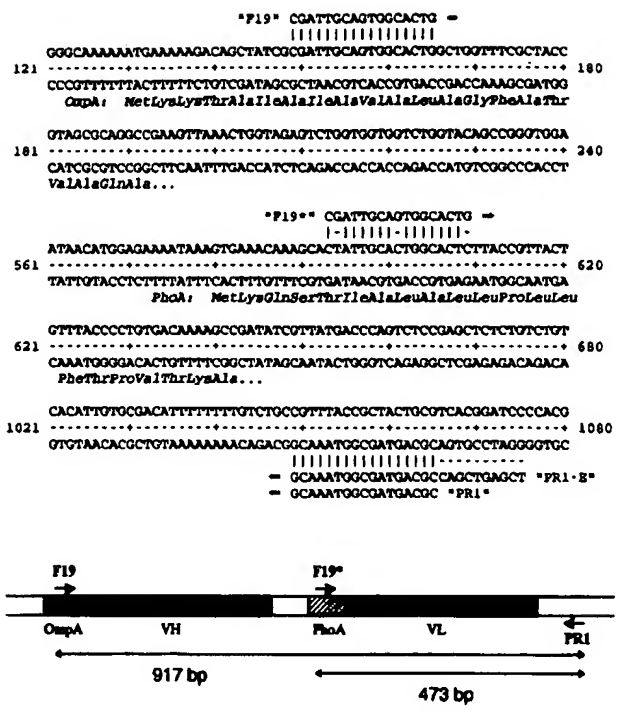

Figure 1. Nucleotide sequences from plasmid pASK30 with matching regions for the primers 'F19', 'PR1', and 'PR1-E' (numbering according to ref. 9). The original priming site for 'F19' lies within the coding sequence for the *Omp*A signal peptide giving rise to a PCR product of 917 base pairs in length with 'PR1' and of 927 base pairs in length with 'PR1-E', respectively. The secondary priming site for 'F19' (marked by a *) with three mismatches is situated in the coding region of the *Pho*A signal peptide leading to a PCR product of 473 base pairs in length with 'PR1'. The formation of the two differently sized amplification products is schematically shown below.

The plasmid pASK30 (9), a vector designed for the functional expression of an antibody F_v fragment in *E. coli*, was chosen as the template nucleic acid. This plasmid codes for the V_H domain and the V_L domain of the myeloma protein McPC603 on a single operon, both fused to bacterial signal sequences, *Omp*A in the first and *Pho*A in the latter case. The primers used in the PCR were originally designed as primers for dideoxy sequencing (11), 'PR1' as an upstream primer matching in the region of the transcription terminator and 'F19' as a downstream primer matching within the coding region for the *Omp*A signal peptide (figure 1).

These primer molecules represent oligodeoxynucleotides perfectly complementary to the template and with lengths close to the lower limit as a substrate for the 3′ to 5′ exonuclease activity expected. Furthermore, 'PR1-E' was used with a ten nucleotide 5′ terminal extension in comparison to 'PR1', which includes a *Pvu*II restriction site and provides no additional complementarity to the template (see figure 1). This oligodeoxynucleotide ought to serve as an example of a primer with the practical properties discussed in the introduction.

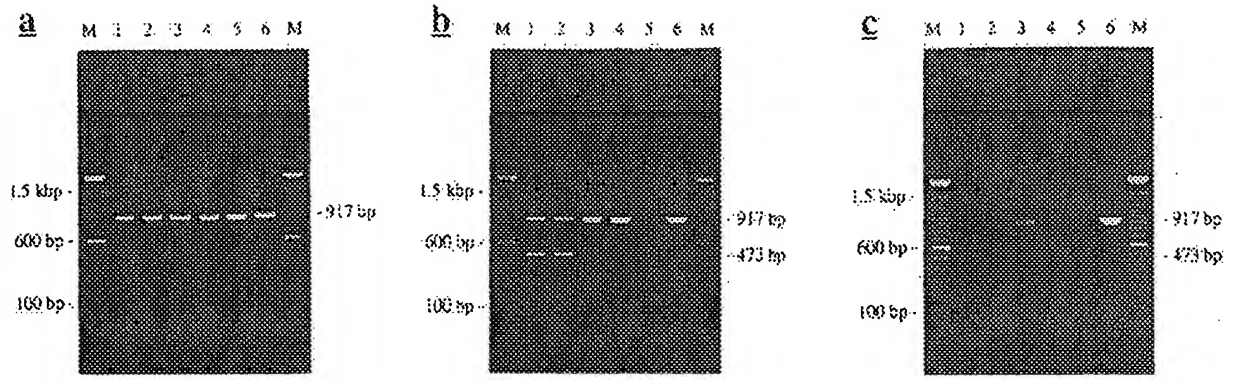

Figure 2. a) PCR reactions were performed using *Taq* DNA polymerase with pASK30 plasmid DNA as the template and the following primer combinations: 'PR1' and 'F19' (lane 1); 'PR1-T' and 'F19' (lane 2); 'PR1-T' and 'F19-T' (lane 3); 'PR1' and 'F19-T' (lane 4); 'PR1-E' and 'F19-T' (lane 5); 'PR1-ET' and 'F19-T' (lane 6). Lane M shows a 100 base pair ladder (Gibco BRL) as the size standard. b) Same as in a) except that *Pfu* DNA Polymerase was used for each PCR reaction. c) Same as in a) except that *Vent* DNA Polymerase was used for each PCR reaction.

Figure 1 also shows a secondary priming site for 'F19' situated in the coding region for the *Pho*A signal peptide with three mismatches, one involving the 3' terminal base. Priming at this site was neither observed during dideoxy sequencing with T7 DNA polymerase (not shown) nor during PCR with *Taq* polymerase (see below). All three primers were synthesized according to standard phosphoamidite solid phase chemistry (5). In addition, analogues of these oligodeoxynucleotides with identical nucleotide sequences were synthesized, carrying single phosphorothioate bonds at their 3' termini and named 'PR1-T', 'F19-T', and 'PR1-ET', respectively (cf. Materials and Methods).

The results from polymerase chain reactions performed with these six primers, pASK30 as the template, and *Pfu* DNA polymerase or *Vent* DNA polymerase are shown in figure 2. In addition, the PCR reactions carried out with *Taq* DNA polymerase instead of the thermostable proofreading DNA polymerases are shown as a control (fig. 2a). In this case, for each primer combination only the expected full length product (917 bp or 927 bp) is observed in a homogenous form and with reproducible yield, thus demonstrating that the effects described below are not due to intrinsic properties of the primer molecules themselves.

The results obtained with *Pfu* DNA polymerase are quite different (fig. 2b). If the 'thio' primer 'F19-T' is used in combination with the 'thio' primers 'PR1-T' or 'PR1-ET' (lanes 3 and 6) a single PCR product with the expected length of 917 or 927 base pairs, respectively, (cf. fig. 1) and with optimum yield is observed. With the 'non-thio' primer 'PR1' instead of 'PR1-T' a comparable result is obtained (lane 4). If, however, 'PR1-ET' is replaced by 'PR1-E', the amount of the PCR product considerably drops (lane 5), thus demonstrating an effect of the protection against exonucleolytic attack on the yield of the polymerase chain reaction for this primer.

Another phenomenon is observed if the 'non-thio' primer 'F19' is used instead of 'F19-T', either in combination with 'PR1' or 'PR1-T' (lanes 1 and 2). Under these circumstances the yield of the expected full length PCR product is slightly decreased and a second band of much smaller size appears with strong intensity on the agarose gel. If a derivative of pASK30 was used as the template DNA where the coding region for the *Pho*A signal peptide was missing, a corresponding side product was not observed (data not shown). Therefore, this product most likely corresponds to the PCR product with an expected size of 473 base pairs caused by the second priming site for 'F19' located downstream to the original matching region (fig. 1). Since it is known that primers with a 3' terminal mismatch provide poor substrates for DNA polymerases (1), the occurance of this non-specific side product can only be explained by the 3' to 5' exonuclease activity of the *Pfu* DNA polymerase. It was already described before that *Pfu* DNA polymerase is able to edit the mismatched 3' end of a primer molecule (3). Sequence analysis revealed that there is no secondary priming site for 'PR1' with comparable quality which explains the absence of a similar phenomenon whether this oligodeoxynucleotide carries a phosphorothioate bond or not.

Figure 2c shows the analogous amplification experiments carried out with *Vent* DNA polymerase. Here the effect observed with the phosphorothioate primers is even more drastic. Only in those cases where both primers are protected against 3' terminal degradation—either 'F19-T' and 'PR1-T' (lane 3) or 'F19-T' and 'PR1-ET' (lane 6)—the expected full length PCR product is observed at all. If 'PR1-T' is used in conjunction with the 'non-thio' primer 'F19', a trace amount of the shorter PCR product is detected (lane 2), probably caused by the secondary priming site for the latter primer as described above. None of the other primer combinations gives rise to a major amplification product, suggesting that the 3' to 5' exonuclease activity for single stranded primer oligodeoxynucleotides exhibited by *Vent* DNA polymerase is significantly more pronounced than it is the case for *Pfu* DNA polymerase.

The results described here clearly demonstrate that the proofreading activity of thermostable DNA polymerases can severely impair with the correct functioning of primers in the amplification of a DNA sequence. This may be the case either by lowering the yield of the PCR product, in some cases even down to no PCR product at all, or by causing non-specific side products resulting from 3' terminal editing of the primer molecule. Both effects can be completely avoided by the introduction of a single phosphorothioate bond at the very 3' terminus of the primer which seems to effectively protect the oligodeoxynucleotide from 3' terminal exonuleolytic attack. This inhibitory effect could not be a priori expected for *Pfu* DNA

polymerase and *Vent* DNA polymerase since earlier studies lead to an inconclusive description regarding the resistance of phosphorothioate linkages towards 3′ to 5′ exonuclease activities (10). So it was reported that the exonuclease activity of *E. coli* DNA polymerase I is inhibited whereas that of T4 DNA polymerase is not.

The strategy outlined in this contribution has already been successfully used during several cloning experiments in the author's laboratory (Skerra, A. et al., to be published), suggesting that the single phosphorothioate bonds introduced into the PCR products neither interfere with restriction enzyme digest (the restriction sites being embedded in the primer sequences) nor with cloning of the nucleic acid itself. In fact, significantly improved amplification efficiencies were obtained routinely both with *Vent* DNA polymerase and with *Pfu* DNA polymerase when using phosphorothioate primer oligodeoxynucleotide analogues, in the case of the latter enzyme essentially matching the performance of *Taq* DNA polymerase. It is therefore believed that this methodology will help the more widespread use of thermostable DNA polymerases with proofreading activity in the amplification of DNA sequences, giving broad access to their enhanced properties compared to *Taq* DNA polymerase.

ACKNOWLEDGEMENTS

The author wishes to thank Christina Wardenberg for technical assistance and Hartmut Michel for continuous encouragement. This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Bloch, W. (1991) *Biochemistry* **30**, 2735–2747.
2. New England Biolabs (1991) *The NEB transcript* 3:1, p5.
3. Lundberg, K. S., Shoemaker, D. D., Adams, M. W. W., Short, J. M., Sorge, J. A., and Mathur, E. J. (1991) *Gene* **108**, 1–6.
4. Lohff, C. J. and Cease, K. B. (1991) *Nucl. Acids Res.* **20**, 144.
5. Kaplan, B. E. (1985) *Trends Biotechnol.* **3**, 253–256.
6. Vu, H. and Hirschbein, B. (1991) *Tetrahedron Lett.* **32**, 3005–3008.
7. Iyer, R. P., Egan, W., Regan, J. B., and Beaucage, S. L. (1990) *J. Am. Chem. Soc.* **112**, 1254–1255.
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
9. Skerra, A., Pfitzinger, I. and Plückthun, A. (1991) *Bio/Technology* **9**, 273–278.
10. Eckstein, F. (1985) *Ann Rev. Biochem.* **54**, 367–402.
11. Tabor, S. and Richardson, C. C. (1987) *PNAS* **84**, 4767–4771.